

Amendments to the Specification

Please replace the paragraph beginning on page 1, line 25 with the following amended paragraph:

Vaccinia topoisomerase binds duplex DNA and forms a covalent DNA-(3'-phosphotyrosyl)-protein adduct at the sequence 5'-CCCTT¹ (SEQ ID NO:1). The enzyme reacts readily with a 36-mer CCCTT (SEQ ID NO:1) strand (DNA-p-RNA) composed of DNA 5' and RNA 3' of the scissile bond. However, a 36-mer composed of RNA 5' and DNA 3' of the scissile phosphate (RNA-p-DNA) is a poor substrate for covalent adduct formation. Vaccinia topoisomerase efficiently transfers covalently held CCCTT (SEQ ID NO:1)-containing DNA to 5'-OH terminated RNA acceptors; the topoisomerase can therefore be used to tag the 5' end of RNA in vitro.

Please replace the paragraph beginning on page 2, line 8 with the following amended paragraph:

Religation of the covalently bound CCCTT (SEQ ID NO:1)-containing DNA strand to a 5'-OH terminated DNA acceptor is efficient and rapid ($k_{rel} > 0.5 \text{ sec}^{-1}$), provided that the acceptor DNA is capable of base-pairing to the noncleaved DNA strand of the topoisomerase-DNA donor complex. The rate of strand transfer to DNA is not detectably affected by base mismatches at the 5' nucleotide of the acceptor strand. Nucleotide deletions and insertions at the 5' end of the acceptor slow the rate of religation; the observed hierarchy of reaction rates is: +1 insertion > -1 deletion > +2 insertion >> -2

deletion. These findings underscore the importance of a properly positioned 5' OH terminus in transesterification reaction chemistry, but also raise the possibility that topoisomerase may generate mutations by sealing DNA molecules with mispaired or unpaired ends.

Please replace the paragraph beginning on page 2, line 24 with the following amended paragraph:

Vaccinia topoisomerase, a 314-amino acid eukaryotic type I enzyme, binds and cleaves duplex DNA at a specific target sequence 5'-(T/C)CCTT¹ (SEQ ID NO:2) (1-3). Cleavage is a transesterification reaction in which the Tp¹N phosphodiester is attacked by Tyr-274 of the enzyme, resulting in the formation of a DNA-(3'-phosphotyrosyl) protein adduct (4). The covalently bound topoisomerase catalyzes a variety of DNA strand transfer reactions. It can religate the CCCTT (SEQ ID NO:1)-containing strand across the same bond originally cleaved (as occurs during the relaxation of supercoiled DNA) or it can ligate the strand to a heterologous acceptor DNA 5' end, thereby creating a recombinant molecule (5-7).

Please replace the paragraph beginning on page 3, line 10 with the following amended paragraph:

Duplex DNA substrates containing a single CCCTT (SEQ ID NO:1) target site have been used to dissect the cleavage and strand transfer steps. A cleavage-religation equilibrium is established when topoisomerase

transesterifies to DNA ligands containing ≥ 18 -bp of duplex DNA 3' of the cleavage site (8-11). The reaction is in equilibrium because the 5'-OH terminated distal segment of the scissile strand remains poised near the active site by virtue of the fact that it is stably base-paired with the nonscissile strand. About 20% of the CCCTT-containing strand is covalently bound at equilibrium (11). "Suicide" cleavage occurs when the CCCTT (SEQ ID NO:1)-containing substrate contains no more than fifteen base pairs 3' of the scissile bond, because the short leaving strand dissociates from the protein-DNA complex. In enzyme excess, >90% of the suicide substrate is cleaved (11).

Please replace the paragraph beginning on page 3, line 26 with the following amended paragraph:

The suicide intermediate can transfer the incised CCCTT (SEQ ID NO:1) strand to a DNA acceptor. Intramolecular strand transfer occurs when the 5'-OH end of the noncleaved strand of the suicide intermediate attacks the 3' phosphotyrosyl bond and expels Tyr-274 as the leaving group. This results in formation of a hairpin DNA loop (5). Intermolecular religation occurs when the suicide intermediate is provided with an exogenous 5'-OH terminated acceptor strand, the sequence of which is complementary to the single strand tail of the noncleaved strand in the immediate vicinity of the scissile phosphate (5). In the absence of an acceptor strand, the topoisomerase can transfer the CCCTT (SEQ ID NO:1) strand

to water, releasing a 3'-phosphate-terminated hydrolysis product, or to glycerol, releasing a 3'-phosphoglycerol derivative (12). Although the hydrolysis and glycerololysis reactions are much slower than religation to a DNA acceptor strand, the extent of strand transfer to non-DNA nucleophiles can be as high as 15-40%.

Please replace the paragraph beginning on page 4, line 17 with the following amended paragraph:

The specificity of vaccinia topoisomerase in DNA cleavage and its versatility in strand transfer have inspired topoisomerase-based strategies for polynucleotide synthesis in which DNA oligonucleotides containing CCCTT (SEQ ID NO:1) cleavage sites serve as activated linkers for the joining of other DNA molecules with compatible termini (13). The present study examines the ability of the vaccinia topoisomerase to cleave and rejoin RNA-containing polynucleotides. It was shown previously that the enzyme did not bind covalently to CCCTT (SEQ ID NO:1)-containing molecules in which either the scissile strand or the complementary strand was composed entirely of RNA (9). To further explore the pentose sugar specificity of the enzyme, we have prepared synthetic CCCTT (SEQ ID NO:1)-containing substrates in which the scissile strand is composed of DNA- and RNA-containing halves. In this way, we show that the enzyme is indifferent to RNA downstream of the scissile phosphate, but is does not

form the covalent complex when the region 5' of the scissile phosphate is in RNA form. Also assessed is the contribution of base-pairing by the 5' end of the acceptor strand to the rate of the DNA strand transfer reaction.

Please replace the paragraph beginning on page 9, line 16 with the following amended paragraph:

Figure 1A-B. Topoisomerase cleavage of DNA-p-RNA and RNA-p-DNA strands (SEQ ID NOS: 3, 4, and 5). (A) The 36-bp substrate used in the cleavage reactions is shown, with the ³²P-labeled scissile phosphate indicated by the filled circle. The segments of the top strand flanking the scissile phosphate, which are either DNA or RNA, are bracketed; the bottom strand is all-DNA. (B) Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 8.0), 0.2 pmol of substrate (either DNA-p-RNA or RNA-p-DNA) and topoisomerase as indicated were incubated at 37° for 10 min. Covalent adduct formation (% of input label transferred to the topoisomerase) is plotted as a function of the amount of enzyme added.

Please replace the paragraph beginning on page 10, line 10 with the following amended paragraph:

Figure 3A-3B. Strand transfer to an RNA acceptor. (A) The structures (SEQ ID NOS: 6 and 7) of the covalent topoisomerase-DNA complex (suicide intermediate) and the 18-mer acceptor strands (DNA or RNA) (SEQ ID NOS: 8 and 9) are shown. (B) Religation reactions were performed

under single-turnover conditions as described under Material and Methods. The extent of relegation (expressed as the percent of input labeled DNA converted to the 30-mer strand transfer product) is plotted as a function of incubation time.

Please replace the paragraph beginning on page 11, line 19 with the following amended paragraph:

Figure 5A-B. 5' DNA-tagging of RNA transcribed by T3 RNA polymerase. (A) The structures of the covalent topoisomerase-DNA donor complex and the RNA acceptor are shown (SEQ ID NOs: 6, 10 and 11). The 5' single-strand tail of the suicide intermediate is complementary to the 18 nucleotides at the 5' end of the T3 transcript. Reaction mixtures contained (per 15 μ l) 50mM Tris-HCl (pH 8.0), 0.3 M NaCl, and 0.1 pmol of 32 P-GMP-labeled T3 transcript. (B) Religation was initiated by the addition of pre-formed topoisomerase-DNA donor (at a 10-fold molar excess over RNA acceptor). Incubation was at 37°C. Aliquots (15 μ l) were removed at the times indicated and quenched immediately by adding SDS and EDTA. The samples were adjusted to 50% formamide, heated for 5 min at 95°C, and electrophoresed through a 12% polyacrylamide gel containing 7 M urea in TBE. Transfer of the 12-nucleotide DNA donor strand to the 5' end of the labeled 36-mer T3 transcript yielded a labeled 48-mer product. Conversion of input 36-mer to 48-mer was quantitated by scanning the gel with a phosphoimager.

Please replace the paragraph beginning on page 12, line 11 with the following amended paragraph:

Figure 6A-C. Kinetics of topoisomerase-catalyzed strand transfer reactions resulting in DNA deletions and insertions. (A) The structure of the pre-formed donor complex is shown at the top of the Figure (SEQ ID NOs: 6 and 7). Religation reactions were performed under single-turnover conditions as described under Materials and Methods. All DNA acceptors were included at a 50-fold molar excess over the input CCCTT-containing substrate. (B) Deletion formation. The structures of the completely base-paired 18-mer acceptor DNA oligonucleotide (open circle) (SEQ ID NO:8), a 17-mer oligonucleotide gap (filled square) (SEQ ID NO:12) and a 16-mer strand (SEQ ID NO:13) that anneals to leave a 2-nucleotide gap (square) are shown. (C) Insertion formation. The structures of the completely base-paired 18-mer acceptor (open circle) (SEQ ID NO:8), a 19-mer oligonucleotide containing 1 extra 5' nucleotide (filled triangle) (SEQ ID NO:14) and a 20-mer acceptor containing 2 extra 5' nucleotides (triangle) (SEQ ID NO:15) are shown. The extent of relegation is plotted as a function of incubation time.

Please replace the paragraph beginning on page 13, line 17 with the following amended paragraph:

Figure 8. Strand transfer to DNA acceptors containing a single 5' base mismatch. Religation reactions were performed under single-turnover conditions as described in Materials and Methods. All DNA acceptors were included at a 50-fold molar excess over the input CCCTT-containing substrate. The structures (SEQ ID NO:8) of the fully complementary 18-mer and the three terminal

nucleotide variants (SEQ ID NOs: 16, 17 and 18) are shown.

Please replace the paragraph beginning on page 13, line 26 with the following amended paragraph:

Figure 9A-B. Kinetics of intramolecular hairpin formation. (A) Hairpin formation without potential for base-pairing. DNA cleavage substrates were prepared by annealing the 5' ³²P-labeled 18-mer scissile strand (SEQ ID NO:19) to a 30-mer complementary strand (filled circle) (SEQ ID NO:20) or an 18-mer complementary strand (circle) (SEQ ID NO:21), the structures of the substrates are shown with the topoisomerase cleavage sites indicated by arrows. Reaction mixtures containing (per 20 μ l) 50 mM Tris HCl (pH 7.5), 0.5 pmol of DNA substrate, and 1 pmol of topoisomerase were incubated at 37°C for 10 min. The mixtures were then adjusted to 0.3 M NaCl. Aliquots (20 μ l) were withdrawn immediately prior to adding salt (time zero) and at various intervals after adding salt; the reactions were quenched immediately by adding an equal volume of stop solution (1% SDS, 95% formamide, 20 mM EDTA). The samples were heat-denatured and electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE. The extent of intramolecular strand transfer (expressed as percent of the input labeled substrate converted to hairpin product) is plotted as a function of time after addition of NaCl. (B) Hairpin formation with potential for base-pairing. The structure of the 18-mer (SEQ ID NO:19)/30-mer cleavage site (SEQ ID NO:22) indicated by an arrow. A reaction mixture containing (per 20 μ l) 50

mM Tris HCl (pH 7.5), 0.5 pmol of DNA substrate, and 1 pmol of topoisomerase was incubated at 37°C for 2 min. The mixtures were then adjusted to 0.3 M NaCl. Aliquots (20 μ l) were withdrawn immediately prior to adding salt (time zero) and at various intervals after adding salt. The extent of intramolecular strand transfer is plotted as a function of time after adding NaCl.

Please replace the paragraph beginning on page 15, line 1 with the following amended paragraph:

Figure 10A-B. Affinity Tagging of RNA Using Vaccinia Topoisomerase. (A) The strand transfer reaction pathway is diagramed in the Figure. The biotinylated DNA substrate (SEQ ID NOS: 23 and 24) which contains a single topoisomerase recognition site is immobilized on the Dynabeads (Dynal) streptavidin solid support. The biotin moiety (indicated by the black square) is introduced at the 5' end of the CCCTT-containing strand via standard protocols for automated oligonucleotide synthesis. The purified vaccinia topoisomerase is reacted with the bead-bound DNA to form a covalent enzyme-DNA donor complex (SEQ ID NOS:25 and 24), as illustrated. Enzyme not bound to DNA is removed by washing the beads with buffer. The strand transfer reaction is initiated by addition of the [³²P]-CMP labeled T7 transcript which is dephosphorylated by prior treatment with alkaline phosphatase. The 5' single-strand tail of the donor complex (SEQ ID NO:26) is complementary to the 12 nucleotides of the 5' end of the T7 transcript. Religation of the covalently held biotinylated DNA strand to the T7 transcript is observed as conversion of the 30-mer RNA to the product of 50

nucleotides (SEQ ID NOS: 27 and 24). The mixture was incubated at 37°C for 15 min. The beads were then recovered by centrifugation, washed, and resuspended in 20 µl of buffer containing 0.8% SDS and 80% formamide. The samples were heated at 95°C for 5 min, centrifuged for 5 min, then the supernatants were electrophoresed through a 12% polyacrylamide gel containing 7M urea in TBE buffer. (B) An autoradiograph of the gel is shown in the Figure. Lane B (Bound) - product of the strand transfer reaction bound to the Dynabeads; lane F (Free) - supernatant from the strand transfer reaction. The positions of the input 30-mer T7 transcript and the 50-mer product are shown at the right.

Please replace the paragraph beginning on page 16, line 6 with the following amended paragraph:

Figure 11. A schematic representation of a method of using DNA-tagged mRNA to obtain full-length gene sequences (SEQ ID NOS: 28-32). Briefly, capped full-length mRNA is isolated by attachment to a solid support, such as by using biotinylated-capped mRNA bound to a magnetic bead conjugated with streptavidin. The isolated mRNA is decapped (using tobacco acid pyrophosphatase) and dephosphorylated (using alkaline phosphatase) then modified with a DNA tag using the methods outlined below. The DNA-tagged mRNA is used to generate first strand cDNA using reverse transcriptase and amplified using PCR. The amplified cDNA is then inserted into a plasmid vector.

Please replace the paragraph beginning on page 17, line 16 with the following amended paragraph:

In an embodiment of the above-described method, the topoisomerase cleavage site is a sequence comprising CCCTT (SEQ ID NO:1). In a preferred embodiment the topoisomerase is a vaccinia topoisomerase enzyme. In a further embodiment the vaccinia topoisomerase enzyme is a modified vaccinia topoisomerase enzyme. In another embodiment the DNA strand having a topoisomerase cleavage site is radiolabelled. In a preferred embodiment the radiolabel is ³²P or a radiohalogen. Means for radio labeling nucleotides are well known in the art (see Ausubel, et. al., Short Protocols in Molecular Biology, 3rd ed., Wiley, 1995; US patent 5,746,997 issued 05/05/98). In another preferred embodiment the DNA strand having a topoisomerase cleavage site is labeled with a biotin moiety or another affinity purification tag such as chitin binding domain, glutathione-S-transferase, and the like. Methods of adding affinity labels to nucleotides are well known in the art (see Carniacci, et. al., Genomics 37: 327-336, 1996; Ausubel, et. al., supra). In an embodiment the topoisomerase-bound DNA intermediate and the acceptor RNA strand are ligated in vitro.

Please replace the paragraph beginning on page 21, line 3 with the following amended paragraph:

In a preferred embodiment of the above-described method, the RNA molecule is dephosphorylated after synthesis or isolation. In another preferred embodiment the dephosphorylation is achieved by treatment of the RNA molecule with alkaline phosphatase. In a preferred embodiment the topoisomerase is a vaccinia topoisomerase

enzyme. In another embodiment the vaccinia topoisomerase enzyme is a modified vaccinia topoisomerase enzyme. In a preferred embodiment the cleavage site comprises CCCTT (SEQ ID NO:1). In another preferred embodiment the method further comprises introducing a biotin moiety or another affinity purification moiety, to the DNA cleavage substrate prior to step (a). In still another preferred embodiment the method further comprises immobilizing the affinity purification tagged DNA cleavage substrate on a solid support prior to step (a). In a preferred embodiment the solid support is a sepharose resin or magnetic beads having an affinity purification material, such as avidin, streptavidin, chitin, glutathione and the like, bound thereto. Methods of preparing such materials are well known in the art. In yet another preferred embodiment the method further comprises purifying a biotinylated 5' end tagged DNA-RNA ligation product by separating the solid support to which the biotinylated 5' end tagged DNA-RNA ligation product is immobilized from a liquid phase comprising unmodified RNA.

Please replace the paragraph beginning on page 24, line 3 with the following amended paragraph:

In an embodiment of the above-described method, the removal of the RNA cap structure is by either of enzymatic treatment of the mRNA with a pyrophosphatase or chemical decapping by periodate oxidation and beta elimination. In a preferred embodiment the pyrophosphatase is tobacco acid pyrophosphatase. In another preferred embodiment the topoisomerase cleavage site is CCCTT (SEQ ID NO:1). In yet another preferred

embodiment the DNA-(N) cleavage substrate has a biotin moiety upstream of the cleavage site and is designated BioDNA-(N). In an embodiment the method further comprises affinity purification of the biotinylated 5' DNA-tagged DNA-RNA ligation product by a binding of the biotin moiety to streptavidin prior to step (e).

Please replace the paragraph beginning on page 28, line 13 with the following amended paragraph:

A DNA tag sequence can be attached to the isolated full-length mRNA using the methods described above. A preferred DNA tag sequence is shown in Figure 11 both as a double stranded DNA cleavage substrate and as a covalent topoisomerase-DNA intermediate. The complementary strand of the topoisomerase-DNA intermediate includes a 3' overhang of from 1 to 4 nucleotides, which can be any mixture of adenine, guanine, cytosine or thymine, designated in the figure as N. These nucleotides will base pair with the first 1 to 4 bases of the 5' end of the isolated mRNA molecule, allowing the covalently attached topoisomerase to catalyze the transesterification reaction which joins the DNA tag to the end of the RNA sequence. The DNA tag sequence comprises a topoisomerase recognition site, preferably CCCTT (SEQ ID NO:1), and in addition may comprise a recognition site for a site-specific restriction endonuclease, such as EcoR1, useful for the subsequent insertion of a cDNA molecule into an expression vector.

Applicants: Stewart Shuman, et al.

Serial No.: 10/666,486

Filed: September 19, 2003

Page 15

Please replace the paragraph beginning on page 38, line 16 with the following amended paragraph:

CCCTT (SEQ ID NO:1)-containing 36-mer oligonucleotides containing a single internal ³²P-label at the scissile phosphate were prepared by ligating two 18-mer strands (synthetic RNA or DNA oligonucleotides) that had been hybridized to a complementary 36-mer DNA strand. The sequence of the proximal CCCTT (SEQ ID NO:1)-containing 18-mer strand was 5'-CATATCCGTGTCGCCCTT (SEQ ID NO:3) as DNA or 5'-CAUAUCCGUGUCCCUU (SEQ ID NO:32) as RNA. The sequence of the distal 18-mer strand was 5' - ATTCCGATAGTGACTACA (SEQ ID NO:4) as DNA or 5' - AUUCCGAUAGUGACUACA (SEQ ID NO:33) as RNA. The distal 18-mer strand was 5' -labeled in the presence of [γ^{32} P] ATP and T4 polynucleotide kinase, then gel-purified. The sequence of the 36-mer strand was 5' - TGTAGTCACTATCGGAATAAGGGCGACACGGATATG (SEQ ID NO:5). The strands were annealed in 0.2 M NaCl by heating at 65°C for 2 min, followed by slow-cooling to room temperature. The molar ratio of the 5' -labeled distal 18-mer to the proximal 18-mer and the 36-mer strand in the hybridization mixture was 1:4:4. The singly nicked product of the annealing reaction was sealed in vitro with purified recombinant vaccinia virus DNA ligase (14, 15). The ligation reaction mixtures (400 μ l) contained 50 mM Tris HCl (pH 8.0), 5 mM DTT 10 mM MnCl₂, 1 mM ATP, 10 pmol of 5' ³²p-labeled nicked substrate, and 160 pmol of ligase. After incubation for 4 h at 22°C, the reactions were halted by the addition of EDTA to a final concentration of 25 mM. The samples were extracted with phenol-chloroform and the labeled nucleic acid was

recovered from the aqueous phase by ethanol precipitation. The 36-mer duplex products were dissolved in TE buffer (10 mM tris HCl, pH 8.0, 1 mM EDTA). Ligation of the labeled 18-mer distal strand to the unlabeled CCCTT (SEQ ID NO:1)-containing 18-mer strand to form an internally labeled 36-mer product was confirmed by electrophoresis of the reaction products through a 17% denaturing polyacrylamide gel. The extents of ligation [36-mer/(36-mer + 18-mer)] were as follows: DNA-p-DNA (88%); DNA-p-RNA (67%); RNA-p-DNA (66%).

Please replace the paragraph beginning on page 40, line 17 with the following amended paragraph:

An 18-mer CCCTT (SEQ ID NO:1)-containing DNA oligonucleotide (5' -CGTGTGCGCCCTTATTCCC) (SEQ ID NO:19) was 5' end-labeled in the presence of [γ ³²P] ATP and T4 polynucleotide kinase, then gel-purified and hybridized to a complementary 30-mer strand to form the 18-mer/30-mer suicide cleavage substrate. Covalent topoisomerase-DNA complexes were formed in a reaction mixture containing (per 20 μ l) 50 mM Tris-HCl (pH 8.0), 0.5 pmol of 18-mer/30-mer DNA, and 2.5 pmol of topoisomerase. The mixture was incubated for 5 min at 37°C. The strand transfer reaction was initiated by adding an 18-mer acceptor strand 5' -ATTCCGATAGTGACTACA (SEQ ID NO:8) (either DNA or RNA) to a concentration of 25 pmol/20 μ l (i.e., a 50-fold molar excess over the input DNA substrate), while simultaneously adjusting the reaction mixtures to 0.3 M NaCl. The reactions were halted by addition of SDS and formamide to 0.2% and 50%, respectively. The samples were heat-denatured and then

electrophoresed through a 17% polyacrylamide containing 7 M urea in TBE (90 mM Tris-borate, 2.5 mM EDTA). The extent of strand transfer (expressed as the percent of input labeled DNA converted to a 30-mer strand transfer product) was quantitated by scanning the wet gel with a phosphorimager.

Please replace the paragraph beginning on page 42, line 14 with the following amended paragraph:

The strand transfer reaction pathway is diagrammed in Figure 10a. The biotinylated DNA Substrate which contains a single topoisomerase recognition site is immobilized on the Dynabeads (Dyna) streptavidin solid support. The biotin moiety (indicated by the black square) is introduced at the 5' end of the CCCTT (SEQ ID NO:1)-containing strand via standard protocols for automated oligonucleotide synthesis. The purified vaccinia topoisomerase is reacted with the bead-bound DNA to form a covalent enzyme-DNA donor complex, as illustrated. Enzyme not bound to DNA is removed by washing the beads with buffer. The strand transfer reaction is initiated by addition of the [³²P]-CMP labeled T7 transcript which is dephosphorylated by prior treatment with alkaline phosphatase. The 5' single-strand tail of the donor complex is complementary to the 12 nucleotides at the 5' end of the T7 transcript. Religation of the covalently held biotinylated DNA strand to the T7 transcript is observed as conversion of the 30-mer RNA to a product of 50 nucleotides.

Please replace the paragraph beginning on page 44, line 21 with the following amended paragraph:

Vaccinia topoisomerase does not bind covalently to CCCTT (SEQ ID NO:1)-containing RNA duplexes; nor does it form a covalent complex on RNA-DNA hybrid duplexes in which one of the two strands is RNA (9). Control experiments showed that the failure to form a covalent adduct on a CCCUU (SEQ ID NO:34)-containing RNA strand was not caused by uracil substitution for the thymine bases in the CCCTT (SEQ ID NO:1) sequence (9). To better understand why vaccinia topoisomerase does not form a covalent complex with all-RNA strands, we prepared 36-bp duplex substrates in which the scissile strand was a tandem RNA-DNA or DNA-RNA copolymer and the noncleaved strand was all-DNA (Fig. 1). These duplexes were uniquely labeled with ³²P at the scissile phosphodiester. The substrate molecules were constructed by annealing two 18-mer oligonucleotides (one of which had been 5' ³²P-labeled) to a complementary 36-mer DNA strand to form a singly nicked duplex. The 5'-labeled 18-mer strand was then joined to the unlabeled CCCTT (SEQ ID NO:1)-strand (or CCCUU (SEQ ID NO:34) strand) in a reaction catalyzed by vaccinia virus DNA ligase. The 36-mer duplex products were isolated and then used as substrates for vaccinia DNA topoisomerase. We will refer to these substrates as DNA-p-DNA, DNA-p-RNA, and RNA-p-DNA, with the labeled phosphate being denoted by p.

Please replace the paragraph beginning on page 45, line 18 with the following amended paragraph:

Transesterification by topoisomerase at the CCCTT (SEQ ID NO:1) site will result in covalent binding of a 3' ³²P-labeled 18-mer oligonucleotide to the enzyme. The extent of covalent complex formation on the DNA-p-RNA substrate in 10 min was proportional to input topoisomerase; 80-85% of the 36-mer strand was transferred to the topoisomerase at saturating enzyme (Fig. 1). The same level of topoisomerase covalently bound less than 1% of the RNA-p-DNA 36-mer strand. Hence, the topoisomerase tolerated RNA substitution downstream of the scissile phosphate, but was impeded from forming the covalent adduct when the CCCTT (SEQ ID NO:1) sequence was in RNA form.

Please replace the paragraph beginning on page 46, line 16 with the following amended paragraph:

The RNA-p-DNA 36-mer was transferred to the topoisomerase, albeit very slowly. After 4 h, 4% of the CCCUU (SEQ ID NO:34)-containing RNA strand was bound covalently to the enzyme (Fig. 2B). An endpoint was not established in this experiment. However, by comparing the initial rate of covalent adduct formation on RNA-p-DNA (0.04% of input substrate cleaved per min) to the amount adduct formed on DNA-p-DNA at the earliest timepoint (12% in 10 sec), it is estimated that RNA substitution of the CCCTT (SEQ ID NO:1)-portion of the substrate slowed the rate of covalent complex formation by about three orders of magnitude.

Please replace the paragraph beginning on page 47, line 2 with the following amended paragraph:

Rejoining of the cleaved strand occurs by attack of a 5' hydroxyl terminated polynucleotide on the 3' phosphodiester bond between Tyr-274 and the CCCTT (SEQ ID NO:1) site. This transesterification step can be studied independent of strand cleavage by assaying the ability of a performed topoisomerase-DNA complex to religate the covalently held strand to a heterologous acceptor strand (5, 11). To form the covalent topoisomerase-DNA donor complex, the enzyme was initially incubated with a suicide substrate consisting of a 5' ³²P-labeled 18-mer scissile strand (CGTGTCGCCCTTATTCCC) (SEQ ID NO:19) hybridized to a 30-mer strand. Cleavage of this DNA by topoisomerase is accompanied by dissociation of the 6-nucleotide leaving group, ATTCC (SEQ ID NO:35). With no readily available acceptor for religation, the enzyme is essentially trapped on the DNA as a suicide intermediate (Fig. 3). In a 5 min reaction in enzyme excess, >90% of the 5' ³²P-labeled strand becomes covalently bound to protein. The strand transfer reaction was initiated by adding a 50-fold molar excess of an 18-mer acceptor strand (either DNA or RNA) complementary to the 5' single-strand tail of the covalent donor complex (Fig. 3), while simultaneously increasing the ionic strength to 0.3 M NaCl. Addition of NaCl during the religation phase promotes dissociation of the topoisomerase after strand closure and prevents recleavage of the strand transfer product. Ligation of the covalently held 12-mer CGTGTCGCCCTT (SEQ ID NO:6) to the 18-mer yields a ³²P-labeled 30 mer (Fig. 4, lane 1). The suicide intermediate transferred 94% of the input CCCTT (SEQ ID NO:1)-containing strand to the 18-mer DNA strand (Fig. 3). The extent of religation at the earliest time point

(5 sec) was 90% of the endpoint value. From this datum a religation rate constant (k_{rel}) of $>0.5 \text{ sec}^{-1}$ was calculated. A k_{rel} value of $\sim 1.3 \text{ sec}^{-1}$ had been determined previously (from experimental values for k_{cl} and K_{eq} at 37°C) (18).

Please replace the paragraph beginning on page 48, line 10 with the following amended paragraph:

Topoisomerase readily ligated the covalently held 12-mer DNA to an 18-mer RNA acceptor to form a 30-mer product (Fig. 4, lane 5). 89% of the input CCCTT (SEQ ID NO:1)-strand was transferred to RNA, with 40% of the endpoint value attained in 5 sec. This datum was used to estimate a rate constant of 0.1 sec^{-1} for single-turnover strand transfer to RNA. Thus, religation to DNA was about 10 times faster than religation to RNA. The slowed rate of RNA religation is likely to account for the observed increase in the cleavage-religation equilibrium constant ($K_{eq} = k_{cl}/k_{rel}$) on the DNA-p-RNA 36-mer.

Please replace the paragraph beginning on page 48, line 22 with the following amended paragraph:

The predicted product of strand transfer to RNA is a 30-mer tandem DNA-RNA strand (5' - CGTGTCGCCCTTAUCCGAUAGUGACUACA) (SEQ ID NO:36) uniquely ^{32}P -labeled at the 5' end. The structure of this molecule was confirmed by analysis of the susceptibility of this product to treatment with NaOH. The labeled 30-mer RNA ligation product was converted nearly quantitatively into a discrete species that migrated more rapidly than the input 18-mer CCCTT(SEQ ID NO:1)-containing

DNA strand (Fig. 4 lane 6). The mobility of this product was consistent with a chain length of 13 nucleotides. The expected ^{32}P -labeled alkaline hydrolysis product of the RNA strand transfer product is a 13-mer (5' -CGTGTCGCCCTTA p) (SEQ ID NO:37). Control reactions showed that neither the ^{32}P -labeled 18-mer scissile strand of the suicide substrate nor the 30-mer product of strand transfer to DNA was susceptible to alkali (Fig. 4, lanes 4 and 2). It is concluded that topoisomerase can be used to ligate RNA to DNA *in vitro*.

Please replace the paragraph beginning on page 49, line 15 with the following amended paragraph:

Practical applications of topoisomerase-mediated strand transfer to RNA include the 5' tagging of RNA transcripts. Bacteriophage RNA polymerases have been used widely to synthesize RNA polymerases have been used widely to synthesize RNA in vitro from plasmid DNA templates containing phase promoters. To test whether such transcripts were substrates for topoisomerase-catalyzed ligation, we constructed a CCCTT(SEQ ID NO:1)-containing suicide cleavage substrate that, when cleaved by topoisomerase, would contain a 5' single-strand tail complementary to the predicted 5' sequence of any RNA transcribed by T3 RNA polymerase from a pBluescript vector (Fig. 5). A 36-nucleotide T3 transcript was synthesized in a transcription reaction containing [$\alpha^{32}\text{P}$] GTP. The RNA was treated with alkaline phosphatase to dephosphorylate the 5' terminus. The topoisomerase-DNA covalent intermediate was formed on an unlabeled suicide substrate. Incubation of the radiolabeled T3 transcript with the suicide intermediate resulted in the conversion

of the 36-mer RNA into a novel species that migrated more slowly during polyacrylamide gel electrophoresis (not shown). The apparent size of this product (48 nucleotides) was indicative of ligation to the 12-mer CCCTT (SEQ ID NO:1) DNA strand. The kinetics of DNA ligation to the T3 transcript are shown in Fig. 5. The reaction was virtually complete within 1 min; at its endpoint 29% of the input RNA had been joined to DNA. No DNA-RNA ligation product was formed in reaction containing a T3 transcript that had not been treated with alkaline phosphatase (not shown).

Please replace the paragraph beginning on page 50, line 18 with the following amended paragraph:

The acceptor polynucleotides used in the experiments described above were capable of hybridizing perfectly with the 5' single-strand tail of the topoisomerase-DNA donor complex. It had been shown previously that the vaccinia virus topoisomerase is capable of joining the CCCTT (SEQ ID NO:1)-strand to an acceptor oligonucleotide that hybridizes so as to leave a single nucleotide gap between the covalently bound donor 3' end and the 5' terminus of the acceptor. Religation across this gap generated a 1 base deletion in the product compared to the input scissile strand (5). The enzyme also catalyzes strand transfer to an acceptor oligonucleotide that, when hybridized, introduces an extra nucleotide between the donor 3' end and the penultimate base-paired nucleotide of the acceptor. Religation in this case will produce a 1 base insertion (5). Deletion and insertion formation in vitro have also been documented for mammalian type I

topoisomerase (19). However, there has been no report of the effects of acceptor strand gaps and insertions on the rate of strand joining by these enzymes.

Please replace the paragraph beginning on page 53, line 1 with the following amended paragraph:

Strand transfer by topoisomerase to a set of 18-mer acceptors that were capable of base-pairing with the 5' tail of the donor complex from positions -2 to -18 (relative to the scissile +1 T:A base pair of the CCCTT (SEQ ID NO:1) element), but which have a base-mismatch at the -1 position immediately 3' of the scissile bond, was examined. The control acceptor, which has a normal -1 A:T base-pair, reacted to completion in 10 sec; 89% of the endpoint was achieved in 5 sec (Fig. 8). DNAs containing T:T, C:T, or G:T mispairs at the -1 position supported the same extent of strand transfer; 77% of the endpoint was attained in 5 sec in each case (Fig. 8). Thus, within the limits of detection of this experiment, mismatch at the -1 position had little effect on the strand transfer reaction. There are clear and instructive differences between the effects of base mismatches versus a single nucleotide deletion on the rate of the strand joining step.

Please replace the paragraph beginning on page 53, line 19 with the following amended paragraph:

In the absence of an exogenous acceptor oligonucleotide, the 5' -OH terminus of the nonscissile strand of the 12-mer/30-mer covalent complex can flip back and act as the

nucleophile in attacking the DNA- (3-phosphotyrosyl) bond (5). The reaction product is a hairpin molecule containing a 12-bp stem and an 18-nucleotide loop. The kinetics of this reaction were examined under single turnover conditions. In the experiment shown in Fig. 9A, 65% of the input CCCTT (SEQ ID NO:1) strand was converted to hairpin product in 3 h. The observed rate constant was $5.7 \times 10^{-4} \text{ sec}^{-1}$. In parallel, the rate of hairpin formation by the covalent complex formed on an 18-bp cleavage substrate (Fig. 9A) was analyzed. In this case, attack by the 5' -OH of the nonscissile strand yielded a hairpin molecule containing a 12-bp stem and a 6-nucleotide loop. 69% of the input CCCTT (SEQ ID NO:1) strand was converted to hairpin product in 10 h. The observed rate constant was $8.2 \times 10^{-5} \text{ sec}^{-1}$. Thus, the 18-nucleotide 5' tail was ~7 times more effective than the 6-mer 5' tail as the attacking nucleophile for strand transfer in *cis*. Note that hairpin formation by these covalent complexes occurs without any potential for base-pairing by the single-strand tails.

Please replace the paragraph beginning on page 54, line 14 with the following amended paragraph:

In order to examine the contribution of base-pairing to the rate of religation, the 5' terminal and penultimate bases of bottom strand of the 18-mer/30-mer substrate to 5' -AT (Fig. 9B) were altered. Now, the 5' -terminal three bases of the bottom strand (5' -ATT) are identical to the 5' -terminal bases of the leaving strand (5' -ATTCCC) (SEQ ID NO:38); hence, the single-strand tail is self-complementary and capable of forming three base-

pairs adjacent to the scissile phosphate. Intramolecular hairpin formation on this DNA was extremely fast; the reaction was complete in 10-20 sec (Fig. 9B). The observed religation rate constant was 0.2 sec^{-1} . By comparing this value to the religation rate constant on the non-complementary 18-mer/30-mer substrate (Fig. 9A), it was surmised that 3 base-pairs accelerated the reaction ~350-fold.

Please replace the paragraph beginning on page 55, line 4 with the following amended paragraph:

The 42-nucleotide 5' ^{32}P -labeled hairpin product was gel-purified and tested as a substrate for covalent adduct formation by the vaccinia topoisomerase. 55% of the input radioactivity was transferred to the topoisomerase polypeptide in 15 sec at 37°C ; an endpoint of 90% transfer was attained in 60 sec (data not shown). The apparent rate constant for cleavage of the hairpin was 0.06 sec^{-1} . Thus, the topoisomerase rapidly and efficiently cleaved a CCCTT (SEQ ID NO:1)-containing molecule in which there were no standard paired bases downstream of the scissile phosphate. The hairpin cleavage rate constant is about one-fifth of k_{c1} on the 18-mer/30-mer suicide substrate, which contains five paired bases of duplex DNA 3' of the CCCTT (SEQ ID NO:1) site.

Please replace the paragraph beginning on page 56, line 5 with the following amended paragraph:

Vaccinia topoisomerase is apparently incapable of binding covalently to CCCUU (SEQ ID NO:34) -containing RNA strands. This is the case whether the CCCUU (SEQ ID NO:34) strand is part of an RNA-RNA or an RNA-DNA duplex (9). It has now been shown that the sugar specificity of the enzyme is attributable to a stringent requirement for DNA on the 5' side of the scissile phosphate, i.e., the CCCTT (SEQ ID NO:1) site must be DNA. Moreover, the CCCTT (SEQ ID NO:1) element must be a DNA-DNA duplex, because earlier experiments showed that a CCCTT (SEQ ID NO:1) strand is not cleaved when annealed to a complementary RNA strand (9). The RNA-DNA hybrid results are informative, because they suggest that the CCCTT (SEQ ID NO:1) site must adopt a B-form helical conformation in order to be cleaved. RNA and DNA polynucleotide chains adopt different conformations within an RNA-DNA hybrid, with the RNA strand retaining the A-form helical conformation (as found in dsRNA) while the DNA strand adopts a conformation that is neither strictly A nor B, but is instead intermediate in character between these two forms (20, 21). Vaccinia topoisomerase makes contacts with the nucleotide bases of the CCCTT (SEQ ID NO:1) site in the major groove (9, 22). It also makes contacts with specific phosphates of the CCCTT (SEQ ID NO:1) site (23). Adoption by the CCCTT (SEQ ID NO:1) site of a non-B conformation may weaken or preclude these contacts.

Please replace the paragraph beginning on page 57, line 11 with the following amended paragraph:

The increase in the cleavage-religation equilibrium constant K_{eq} ($= k_{cl}/k_{rel}$) on the DNA-p-RNA substrate can be explained by the finding that the rate of single-turnover RNA religation $k_{rel(RNA)}$ is about one-tenth of $k_{rel(DNA)}$. Nonetheless, the extent of religation to RNA is quite high, i.e., ~90% of the input CCCTT (SEQ ID NO:1) strand is religated to an 18-mer RNA acceptor strand in a 2 min reaction. It is shown that a CCCTT (SEQ ID NO:1)-containing DNA strand can be rapidly joined by topoisomerase to a transcript synthesized in vitro by bacteriophage RNA polymerase; ~30% of the RNA is transferred to the DNA strand in a 2-5 min reaction. This property can be exploited to 5' tag any RNA for which the 5' terminal RNA sequence is known, i.e., by designing a suicide DNA cleavage substrate for vaccinia topoisomerase in which the nonscissile strand is complementary to the 5' sequence of the intended RNA acceptor. Some practical applications include: (i) ^{32}P -labeling of the 5' end of RNA and (ii) affinity labeling the 5' end of RNA, e.g., by using a biotinylated topoisomerase cleavage substrate. A potential advantage of topoisomerase-mediated RNA strand joining (compared with the standard T4 RNA ligase reaction) is that ligation by topoisomerase can be targeted by the investigator to RNAs of interest within a complex mixture of RNA molecules.

Please replace the paragraph beginning on page 61, line 19 with the following amended paragraph:

The use of a DNA-tagged RNA to clone gene sequences was evaluated using 96 base test RNA fragment of known sequence (GGG AGA CCC AAG CTC GCC CGG TTC TTT TTG TCA AGA

Applicants: Stewart Shuman, et al.

Serial No.: 10/666,486

Filed: September 19, 2003

Page 29

CCG ACC TGT CCG GTG CCC TGA ATG AAC TGC AGG ACG AGG CAG
CGC GGC TAT CGT GGC TGG) (SEQ ID NO:39). This test RNA
was synthesized using a T7 Invitrotranscription kit from
Ambion Co. using protocols supplied by the manufacturer.

Please replace the paragraph beginning on page 61, line 26
with the following amended paragraph:

A topoisomerase-DNA intermediate was generated as
follows: 25 µl of streptavidin conjugated Dynabeads
(Dynal) were washed twice with 25 l of 2X B&W buffer (10
mM Tris pH 7.5, 1 mM EDTA, 2 M NaCl) in an eppendorf tube
then resuspended in 50 µl 1X B&W buffer. 1.5 µg of a
biotinylated oligo (TOPOB1) and .75 µg of two annealing
oligos (TOPOP2, TOPOP3) were added to the beads and
heated to 70° C for 5 minutes, then cooled on ice for 2
minutes. The beads were then washed twice with 25 µl
each of NEB #1 buffer (New England Biolabs - 10mM Bis
Tris Propane-HCl, 10mM MgCl₂, 1mMDTT pH7.0 @ 25°) to
remove any unannealed oligonucleotides. The
oligonucleotides were synthesized by Dalton
Biochemicals(Canada) and had the following sequences:

TOPOB1 - 5' B-GTTTTGGCTCCCATATACGACTCGCCCTTNTTCCGATAGTG
(SEQ ID NO:40)

TOPOP2 - 5'-NAAGGGCGAGTC (SEQ ID NO:41)

TOPOP3 - 5'-CACTATCGGAA. (SEQ ID NO:42)

The 5' end of TOPOB1 was biotinylated by using a biotinylated guanine nucleotide during that round of automated synthesis.

Please replace the paragraph beginning on page 63, line 6 with the following amended paragraph:

The DNA-tagged RNA bound beads were next washed twice with 1X RT buffer (cDNA Cycle Kit, Invitrogen, Carlsbad, CA, cat. # L1310-01), primed with RT96 (synthesis of first strand) and PCR performed using the cDNA Cycle Kit according to the manufacturer's instructions and primers PCR96 and PCR53.

RT96 - 5'-CCACGATAGCCGCGCT (SEQ ID NO:43)

PCR96 - CGTCCTGCAGTTCATTCAG (SEQ ID NO:43)

PCR53 - GGCTCCCATATACGACTC (SEQ ID NO:45)

Following the section entitled "Abstract of the Disclosure" on page 77 of the specification and before the Figures, please insert the Sequence Listing attached hereto as **Exhibit B**.